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Gestational-Dependent Changes in the Expression of Signal Transduction and Contractile Filament-Associated Proteins in Mouse Myometrium

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Abstract

Objectives—The timely onset of powerful uterine contractions during parturition involves the integration of many signaling pathways, the protein components of which may be translationally regulated throughout gestation. We have utilized the pregnant mouse model to examine gestational-dependent changes in the expression of several proteins implicated in the coordination of myometrial excitation-contraction coupling: caveolins 1-3, rho-associated kinases (ROK α and β), α -actin, MLC₂₀, and h-caldesmon.

Methods—Protein expression was examined by Western blotting of myometrial homogenates from nonpregnant mice (NP) and compared to that from pregnant mice on gestational days 12, 15, 17, and 19 (term = day 19).

Results—All protein expressions were unchanged during the estrous cycle. α -Actin was found to be invariant throughout pregnancy. The expressions of caveolin-1, -2 and -3, when compared to α -actin expression, also did not change significantly with mid- to late pregnancy. h-caldesmon: α -actin, ROK α : α -actin, and ROK β : α -actin ratios were, however, significantly elevated on day 19, whereas MLC₂₀: α -actin was significantly down-regulated on day 12 to day 19. Consistent with elevated ROK expression at term, the ROK inhibitor Y27632 gave a greater reduction of thromboxane-stimulated contractions in myometrium from day 19 mice compared to NP mice.

Conclusions—These data suggest that in mouse myometrium there is a dynamic regulation of the expression of several proteins implicated in contractile signal integration. This may be important for regulating (1) relative uterine quiescence to ensure pregnancy progression and (2) priming the tissue for requisite contractile effort at term.

Keywords

Myometrium; protein expression; caveolins; ROK; contractile filament proteins

Parturition is associated with the integration of many signaling pathways leading to coordinated activation of uterine contractions. That these processes are activated in a timely manner close to term is crucial to prevent premature labor. Yet uterine smooth muscle is far from static during pregnancy; although there is a relative in vivo contractile quiescence compared to term, there are profound alterations in the size and physiology of myometrial cells that necessitate a remodeling of cell morphology and behavior. It follows that there must be mechanisms available to regulate the dynamic behavior of uterine smooth muscle

cells throughout gestation. One such process may be the ability to regulate the expression of proteins involved in the regulation of uterine contractile activation.

Uterine smooth muscle contractility is regulated primarily by an elevation of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) resulting in an increased phosphorylation of the regulatory light chains of myosin (MLC₂₀) via activation of myosin light chain kinase.^{1–3} In addition to such thick myosin filament regulatory events, recent evidence implicates thin actin filament processes in uterine function. Remodeling of actin filaments is likely to play an important role in the adaptation to cellular hypertrophy in pregnancy but also in the regulation of contractility itself; inhibitors of actin filament polymerization reduce agonist-dependent contractions in a variety of smooth muscles including the uterus.^{4,5} In addition, changes in the expression of the thin filament protein *h*-caldesmon have been correlated with gestation in rat and human myometrium and implicated in the regulation of uterine contractility.^{6,7}

Receptor-coupled contraction of uterine smooth muscle is regulated not only by elevations of $[\text{Ca}^{2+}]_i$, but also by intracellular signaling cascades that sensitize the myofilaments to the activating $[\text{Ca}^{2+}]_i$. A major pathway for agonist-induced Ca^{2+} sensitization is activation of the small G-protein rhoA and its downstream effector rho-associated kinase (ROK). ROK activation has been suggested to increase net force production, at least in part, by reducing the activity of the targeting subunit of myosin light chain phosphatase and thereby accentuating MLC₂₀ phosphorylation.^{8,9} The use of a pharmacological inhibitor of ROK, Y-27632, has resulted in reduction of agonist-induced Ca^{2+} sensitization of uterine force.^{10–13}

Recruitment of rhoA/ROK to the plasma membrane of smooth muscle cells is a crucial step for agonist-induced Ca^{2+} sensitization to occur.^{14,15} Recent evidence in animal studies suggests that specialized membranous invaginations—caveolae—may be involved in this relocalization. Caveolins are proteins that are integral to caveolae and, in vitro, bind to and affect the activity of many signal-transducing molecules. A short cytoplasmic region of caveolin, termed the scaffolding domain peptide, is a key region for these interactions and introduction of this peptide to uterine smooth muscle cells abrogates receptor-coupled signaling protein translocations.¹⁶ Caveolins, therefore, may be important mediators of uterine receptor-coupled signaling events.¹⁷

Preparation for contractile activation at term has been suggested to encompass the translational regulation of many contractile-associated proteins (CAPs).^{18,19} It thus becomes important when considering the possible contribution(s) of particular proteins to uterine function that any translational changes with gestation are fully elucidated. In the present study, therefore, we examined if there were gestational-dependent changes in the expression of the aforementioned proteins implicated in the scaffolding and integration of uterine contractile signals. As such, we compared the expressions of caveolins, ROKs, actin, MLC₂₀, and *h*-caldesmon in the same samples of uterine smooth muscle isolated from nonpregnant mice and mice at different stages of pregnancy.

MATERIALS AND METHODS

Animals

Age-matched adult ICR-(CD1) mice (Harlan UK, Shaw's Farm, Blackthorn, Bicester, Oxon, UK) were killed by cervical dislocation according to schedule 1 of UK Home Office guidelines. Uterine horns were excised and opened up along the mid-line. In pregnant mice, the placentae and fetuses were removed and pups decapitated. Longitudinal strips of myometrium were isolated from the tissue and freed of any endometrium, under a dissecting microscope, and cleaned of any blood by rinsing in Krebs solution (154 mM NaCl, 5.4 mM

KCl, 1.2 mM MgSO₄, 1.6 mM CaCl₂, 5.5 mM glucose, 10 mM 3-[N-Morpholino]propanesulfonic acid [MOPS], pH 7.4). The strips were either snap frozen in liquid N₂ and stored at -80C until use, or prepared immediately for contractile studies.

Western Blot Analysis

Approximately 10–15 mg of frozen tissue was homogenized at 4C until completion in clean glass tissue grinders (Kontes Glass Company, Vineland, NJ) in ~250 µL of homogenization buffer (10% glycerol [vol/vol], 4% sodium dodecyl sulfate [SDS; wt/vol], 1% Triton X-100 [vol/vol], 20 mM MOPS, pH 7.0, 10 mM dithiothreitol, 20 mM α-glycerophosphate, 5.5 µM leupeptin, 5.5 µM pepstatin, 0.0205 TIU/mL apro-tinin, 20 µM phenylmethylsulfonyl fluoride [PMSF]). Each homogenate was centrifuged at 16,060 × *g* for 5 minutes, the supernatants decanted, and the protein concentration estimated using Bio-Rad DC Protein Assay reagents (Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK) and a bovine serum albumin (BSA) standard curve. BSA absorbances (750 nm) were analyzed in duplicate in a Jenway Genova spectrophotometer (Jenway Ltd, Dunmow, Essex, UK), while samples of unknown protein concentration were analyzed in quintuplicate. Following the addition of 2X Laemmli Sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris-HCl, pH 6.8) in a 1:1 ratio, the samples were heated at 100C for 10 minutes, and stored at -20C until use. Samples were prepared on the same day with identical solutions from tissue of nonpregnant mice (NP) and pregnant mice of gestational days 12, 15, 17, and 19 (day 18 to 19 with term = day 19; day 19 groups include tissues isolated from mice late on gestational day 18, or on day 19 as term pregnant mice tended to litter overnight on day 18 to 19 or on day 19). In a separate set of experiments, samples were prepared from NP mice at different stages of the estrous cycle as determined by histologic examination of vaginal smears.²⁰

SDS-electrophoresis was performed with a Mini-Protean II electrophoresis system (Bio-Rad Laboratories). A discontinuous polyacrylamide gel system was used consisting of a 4% stacking gel (0.1% SDS [wt/vol], 0.05% ammonium persulfate, 1:2000 N,N,N',N'-Tetramethylethylenediamine [TEMED], 0.125M Tris-HCl, pH 6.8) and 10% separating gel (0.1% SDS [wt/vol], 0.05% ammonium persulfate, 1:2000 TEMED, 0.375 M Tris-HCl, pH 8.8). Tissue homogenates were electrophoresed in Tris/glycine running buffer at 40 mA/gel for 35–40 minutes. For Western blotting the gels and methanol-activated polyvinylidene fluoride (PVDF) membranes were equilibrated in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 30 minutes. Electrophoretic transfer of protein to the PVDF membrane was performed at 50 V/gel for 1.5 hours at room temperature.

Blocked membranes (5% dried milk in Tris-buffered saline [TBS], 1 hour) were incubated overnight at 4C in TBS with 1% dried milk and one of the following monoclonal antibodies: 1:1000 anti-caveolin-1, 1:500 anti-caveolin-2, 1:500 anti-ROKα, 1:250 anti-ROKβ (BD Transduction Laboratories, Oxford, UK), 1:100 anti-caveolin-3 (Santa Cruz Bio-technology Inc, Santa Cruz, CA), 1:2500 anti-MLC₂₀, or 1:1000 anti-caldesmon (Sigma-Aldrich Company Ltd, Poole, Dorset, UK). Washed membranes were then incubated with 1:2000 goat anti-mouse IgG Peroxidase Conjugate (Calbiochem-Novabiochem Ltd, Nottingham, UK) secondary antibody for 1 hour at room temperature. Membranes were stained with enhanced chemiluminescent substrate (ECL; Super Signal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL) and visualized on X-OMAT AR imaging film (Kodak, Rochester, NY). Membranes either (1) cut at appropriate molecular weights following electrophoretic transfer, or (2) stripped of originally bound primary antibody, were reprobed with 1:100,000 anti-α smooth muscle actin monoclonal antibody (Sigma-Aldrich), incubated with secondary antibody, and stained with ECL as earlier. Predicted molecular weights for antibody-probed proteins were caveolin-1 22 kd, caveolin-2 22 kd, caveolin-3 21 kd, ROKα 180 kd, ROKβ 160 kd, α-actin 44 kd, MLC₂₀ 20 kd, *h*-caldesmon 130 kd.

The anti-ROK β antibody consistently resulted in the appearance of a doublet: one band of the anticipated molecular weight of 160 kd and another band of molecular weight ~200 kd. The 160-kd band was the same size as the band obtained with a positive control sample (mouse kidney lysate; BD Transduction Laboratories). Also, the larger band colocalized on naphthol blue stained membranes with myosin heavy chain (205 kd) and is likely to represent a nonspecific cross-reactivity with this protein. As such, the lower 160-kd band alone was taken to represent ROK β .

Equal protein loadings (10 or 20 μ g per lane) of total homogenate were used. In one series of experiments, uterine samples from NP mice in pro-estrus or estrus were run on gels together with samples of mice in di-estrus to allow for relative protein quantifications between different stages of the estrous cycle. As the proteins of interest did not vary in expression during the estrous cycle (see later), in another series of experiments, uterine samples from each selected gestation were run on one gel together with a sample from a nonpregnant mouse to allow for relative protein quantifications between gestational states. This process ensured identical treatment of samples from different conditions throughout the entire detection procedure. Protein quantification was determined by densitometric scanning (GS-700 and Molecular Analyst; Bio-Rad Laboratories) of imaging films. Scanning of films exposed to ECL-stained membranes for different periods of time ensured that ECL signals had not saturated. Comparison between conditions for any one protein of interest was performed as follows: all densitometric raw data values for tissues of pregnant mice were compared to the value obtained for the same amount of NP homogenate (normalized to 1.0) on the same gel/membrane. In addition, this allowed an assessment of the amounts of any one protein of interest relative to α -actin from the same membrane. For example, MLC₂₀ expression on day 19 was assessed as x-fold of NP and α -actin expression on day 19 was determined as y-fold of NP. Therefore, a value of MLC₂₀: α -actin expression on day 19 versus NP of x-fold/y-fold was obtained. In such a manner, a ratio of the amounts of each protein of interest, relative to α -actin, was calculated for each gestational age compared with NP conditions.

Contractile Studies

Small strips of myometrium (~5 mm length x 1 mm wide x 1 mm thick) were tied at each end with silk thread and mounted in a standard organ bath chamber (Linton Instruments, Palgrave, Norfolk, UK). One tissue end was tied to a fixed hook, the other to a TSD105A tension transducer (Linton Instruments) and equilibrated in physiological salt solution (PSS; composition [mM]: NaCl 119, KCl 4.7, MgSO₄·7H₂O 2.4, NaHCO₃ 25, KH₂PO₄ 1.18, K₂EDTA 0.03, glucose 6.05, CaCl₂·2H₂O 1.6 at pH 7.4) at 37°C. For experiments to determine the absolute contractile force (taken as force per cross sectional area, mN/mm²) from myometria of NP or pregnant mice, tissues were stimulated at initial slack length with high K⁺ solution (60 mM KCl substituted for NaCl) for 5 minutes. After washout with PSS, the tissues were stretched by known increments of 0.25 mm using the calibrated micro-manipulator attached to the tension transducer. After 10 minutes, the tissues were stimulated again by high K⁺ solution. This procedure was repeated at several tissue lengths to allow construction of a length-tension curve and determination of the length that allowed for maximal force production. At this length, the integrated active tension in response to 5 minutes high K⁺ stimulation was recorded and compared between myometria from pregnant animals of different gestations (days 12, 15, 17, 19) and that of NP mice. For other experiments to investigate the role of ROK to contractile activation, myometrial strips isolated from NP and day 19 pregnant mice were stimulated with the thromboxane mimetic U46619 (10 μ M) for 10–20 minutes on two or three separate occasions to ensure reproducible contractile responses. Paired tissues, run in parallel in separate chambers, were then further exposed to (1) U46619 in combination with the ROK inhibitor Y-27632 (10

μM) until maximum effect on contractility was observed; (2) U46619 alone to serve as an additional time control to ensure there was no tachyphylaxis to consecutive U46619 exposures. The maximal effect of Y27632 was expressed relative to the peak force measured for an equivalent time point in the presence of U46619 alone.

Materials

Materials other than those previously mentioned were purchased from Sigma-Aldrich, except the following: NaCl, CaCl₂, NaHCO₃, KHPO₄, K₂EDTA, acetic acid, and methanol (VWR International Ltd, Lutterworth, Leicestershire, UK); acrylamide/bis acrylamide and filter paper (Bio-Rad Laboratories); PVDF membrane (Scheicher & Schuell, Dassel, Germany); and U46619 and Y-27632 (Calbiochem-Novabiochem Ltd, UK).

Statistics

Differences in absolute contractile force of myometrial strips with pregnancy were analyzed by one-way analysis of variance (ANOVA), followed, if appropriate, by Dunnett's post-hoc tests for differences between groups. Similarly, differences in normalized protein and selected protein:α-actin ratios were also determined by one-way ANOVA and, if appropriate, Dunnett's post-hoc test for differences between groups. Contractile amplitudes of myometrial strips following the application of Y-27632, normalized to amplitudes pretreatment, or of strips isolated from pregnant mice relative to those of NP animals, were analyzed by paired and unpaired Student *t* test, respectively. Significance was indicated by *P* < .05. All results are expressed as mean ± SEM. *N* = number of mice sacrificed for a given gestational day (or nonpregnancy); *n* = number of times that myometrial samples from a particular grouping (NP, day 12 etc) were electrophoresed and immunoblotted. For example, the same myometrial samples were often immunoblotted for our reference protein α-actin on more than one occasion and this is denoted by *n*; therefore, for each particular grouping (NP, day 12 etc) the *n* were meaned per *N*.

RESULTS

The Comparison of Absolute Contractile Force Between Myometria of Nonpregnant and Pregnant Mice

Changes noted, if any, in the expression of signal transduction or contractile filament-associated proteins with pregnancy may have implications for the capacity for force production of the myometrial tissue. As such, we wished to examine what alterations, if any, in the absolute force production of myometrial tissue occurred between non-pregnancy and different stages of pregnancy. As illustrated in Figure 1, there was a progressive increase in the absolute force (expressed as integrated force per cross-sectional area for 5-minute exposure to high K⁺ depolarizing solution) of myometrial tissue from pregnant mice when compared to NP mice such that the force produced on days 15, 17, and 19 of pregnancy was significantly greater than that of NP mice. The absolute force produced by myometria of pregnant mice on gestational days 15, 17, and 19, respectively, was 1.81 ± 0.25-, 2.48 ± 0.41-, and 2.90 ± 0.65-fold of that of myometria from NP mice (Figure 1; *N* = 4).

Myometrial α-Actin Protein Expression During the Estrous Cycle

The proportion of α-actin expressed in myometrial tissue homogenates did not change during the estrous cycle of NP mice as depicted in Figure 2. The proportions of α-actin in pro-estrus and estrus mice were 1.15 ± 0.16-fold and 1.05 ± 0.12-fold, respectively, of di-estrus mice (Figure 3A, *N* = 4, *n* = 4). Thus, this enabled a comparison of other proteins of interest during the estrous cycle to be made with α-actin.

Effect of the Estrous Cycle on the Expression of Caveolins, ROKs, *h*-caldesmon, and MLC₂₀

As shown in the examples of Figure 2, the patterns of expression of caveolin 1–3, ROK α , or ROK β , MLC₂₀, or *h*-caldesmon did not change during the estrous cycle. When normalized to the amount of α -actin from the same blots, the caveolin-1: α -actin ratio was 0.92 ± 0.15 in pro-estrus or 1.04 ± 0.23 in estrus of that in di-estrus (Figure 3B, $N = 4$, $n = 4$). Similarly, the caveolin-2: α -actin and caveolin-3: α -actin ratios were, respectively, 0.91 ± 0.13 and 1.06 ± 0.15 in pro-estrus, or 0.91 ± 0.15 and 1.16 ± 0.26 , in estrus of that in di-estrus (Figure 3C–D).

Neither ROK α nor ROK β was significantly altered during the estrous cycle. The ROK α : α -actin and ROK β : α -actin ratios were, respectively, 0.99 ± 0.12 and 1.01 ± 0.16 in pro-estrus, or 1.09 ± 0.24 and 1.15 ± 0.06 in estrus, of that in di-estrus (Figure 3E–F).

Similarly, myometrial expressions of *h*-caldesmon and MLC₂₀ were invariant during the estrous cycle. The MLC₂₀: α -actin and *h*-caldesmon: α -actin ratios were, respectively, 0.97 ± 0.17 and 0.91 ± 0.06 in pro-estrus, or 1.06 ± 0.15 and 1.05 ± 0.20 in estrus, of that in di-estrus (Figure 3G–H).

Myometrial α -Actin Protein Expression During Pregnancy

As illustrated in the representative examples of Figures 4–7, the proportion of α -actin expressed in tissue homogenates did not change between NP mice and pregnant mice at different gestations. On average, the α -actin proportions in day 12, 15, 17, and 19 samples were 0.96 ± 0.04 -fold, 0.95 ± 0.03 -fold, 1.01 ± 0.05 -fold, and 1.08 ± 0.04 -fold respectively of NP samples ($N = 12$; $n = 36$). Hence, this allowed a comparison of other proteins of interest throughout gestation to be made with α -actin.

Effect of Pregnancy on Expression of Caveolins

Myometrial samples from NP and pregnant mice were examined for the expressions of each of the main mammalian caveolin isoforms: caveolin-1, caveolin-2, or caveolin-3. As illustrated in Figure 4, caveolin-1 and -2 expressions, relative to total protein content, showed a trend to increase with pregnancy. However, when compared to the changes in α -actin expression from the same samples (Figure 4B), with 12 mice in each gestational group, the caveolin-1: α -actin ratios were not significantly elevated (being 1.06 ± 0.06 -fold, 1.40 ± 0.20 -fold, 1.44 ± 0.20 -fold, and 1.31 ± 0.12 -fold of NP, respectively; Figure 4C). Similarly, the expression of caveolin-2 when normalized to the α -actin content of each sample, showed no significant alterations with gestation (Figure 4D–F; $N = 12$; $n = 12$). Although there appeared a trend for caveolin-3 to decrease with pregnancy, the caveolin-3: α -actin ratios were not significantly different from NP (Figure 4G–I; $N = 10$, $n = 10$).

Effect of Pregnancy on Expression of ROKs

Myometrial samples from NP and pregnant mice were examined for the expressions of the main mammalian ROK isoforms: ROK α or ROK β . The expression of ROK α was significantly greater in uterine samples of pregnant mice than NP mice (Figure 5A). For example, the ROK α : α -actin ratios in day 12, 15, 17, and 19 samples were 2.03 ± 0.12 -fold, 2.13 ± 0.20 -fold, 2.39 ± 0.21 -fold, and 2.43 ± 0.24 -fold, respectively, of NP samples ($N = 6$; $n = 6$; Figure 5C). Similarly, myometrial ROK β expression increased with pregnancy such that on day 19 there was a significant elevation of ROK β , relative to total protein content, compared to tissues of NP mice. The ROK β : α -actin ratio on day 19 was 1.86 ± 0.25 -fold of samples from NP mice ($N = 6$; $n = 6$; Figure 5F).

Effect of Pregnancy on Expression of MLC₂₀

As illustrated in Figure 6A, the expression of MLC₂₀ in tissues of pregnant mice was significantly lower than that of NP mice. Thus, the respective MLC₂₀: α -actin ratios of myometria from day 12, day 15, day 17, and day 19 mice were only 0.68 ± 0.03 -fold, 0.63 ± 0.06 -fold, 0.64 ± 0.05 -fold, and 0.59 ± 0.07 -fold ($N = 6$; $n = 6$) of NP mice (Figure 6C).

Effect of Pregnancy on Expression of *h*-caldesmon

Myometrial expression of *h*-caldesmon in pregnant mice increased significantly on day 17 and day 19 compared to NP mice (Figure 7A). The *h*-caldesmon: α -actin ratios in day 17 and day 19 mice were 1.80 ± 0.08 -fold and 1.81 ± 0.22 -fold, respectively, of NP mice ($N = 4$; $n = 4$; Figure 7C).

Effect of the ROK Inhibitor Y-27632 on U46619-Induced Contractility of Nonpregnant and Late-Pregnant Mouse Myometria

Myometrial strips from NP and late-pregnant (day 18–19) mice were stimulated with the thromboxane mimetic U46619 (10 μ M) until the contraction profile stabilized. The subsequent application of 10 μ M Y-27632 induced a significantly greater relaxation of contractile amplitude of pregnant tissues (Figure 8B) than on those isolated from NP mice (Figure 8A). Thus, during exposure to Y-27632, the contractile amplitudes of NP and pregnant strips fell to 0.80 ± 0.05 -fold ($N = 5$) and 0.56 ± 0.06 -fold ($N = 6$), respectively, of that prior to the application of Y-27632.

DISCUSSION

Pregnancy is often thought of as a situation whereby physiologic adaptations occur to (1) promote an environment to nurture and sustain the growing fetus and (2) prepare the myometrium for the powerful and coordinated contractile activity required at term. We hypothesized that regulation of the latter process may involve changes in proteins implicated in uterine excitation-contraction coupling. We report the influence of mid- to late-pregnancy on the expression patterns in mouse myometrium of several proteins implicated in excitation-contraction coupling of the uterus. Our findings indicate that while α -actin and caveolin expression remained constant relative to total protein, the expressions of the other proteins examined were significantly altered by gestational age. These data indicate that the expressions of proteins suggested to be important for the coordination of excitation-contraction coupling in the uterus are dynamically regulated with gestation. The absolute force production of mouse myometrial tissue also progressively increased with pregnancy and the possible physiological implications of the protein expression patterns are discussed in this light below. The mouse was an appropriate species to investigate this as (1) it allows for assessment of predetermined, timed gestational changes in myometrial protein expression that is not possible with human myometrial samples; (2) the inbred mice offer less genetic and environmental variability than humans; and (3) the approach of focusing an investigation on the possible changes in expression patterns of proteins already suggested to be important for myometrial physiology is a useful complement to more general mRNA screening procedures afoot in the post-genomic age.^{21–24}

The expressions of numerous genes are known to be under the control of estrogen response elements. As such, one might anticipate that any physiological condition exhibiting changes in circulatory or local estrogen levels may lead to altered expression of estrogen-responsive genes and/or proteins. Indeed, previous works have reported that the expression of caveolin protein in rat uterus and bladder is regulated by chronic estrogen levels.^{25,26} However, we found there to be no difference in the expression of α -actin, caveolins, ROKs, *h*-caldesmon, or MLC₂₀ proteins during the estrous cycle of normal NP mice. Fortuitously, therefore, it

was possible to compare the effects of gestation on myometrial expression of these proteins compared to the nonpregnant situation. A recent study also of note is that which examined the expression of more than 8000 genes in the mouse uterus with the estrous cycle.²³ Although 102 genes were reported to be up- or down-regulated with the estrous cycle, these did not include genes encoding the eight proteins of interest to our study.

Relative to total homogenate protein, α -actin content was found not to be altered in mid- to late-gestation compared to tissue from NP mice. A similar situation has been reported for total actin and myosin content of human myometrium from late term women when compared to nonpregnant women.³ Thus, although total myometrial protein content increases due to cell hypertrophy with pregnancy, the amount of α -actin appears to be regulated in proportion to the total myometrial protein. This serves the useful purpose of offering a comparison of invariant α -actin with any other myometrial protein of interest whose expression may change with gestation.

Caveolins, proteins integral to membranous flask-shaped invaginations termed caveolae, interact with a variety of downstream signaling molecules including those activated by uterotonic agents.^{16,17} In rat myometrium, caveolin-1 expression has been suggested to be gestational-dependent and, as mentioned earlier, under the control of chronic estrogen levels.²⁵ However, in mouse myometrium, no significant changes in the pattern of expression were observed with pregnancy. The reason for the difference between these results and those of the rat²⁵ is unknown. The expressions of many myometrial proteins in rodents are known to be under the control of the estrogen:progesterone ratio, which increases markedly in the last 24 hours before labor and both rats and mice share luteolysis as the mechanism precipitating the fall in progesterone and labor onset.^{27,28} In the mouse, our data suggest that caveolin levels are not dramatically altered with pregnancy and, therefore, it is unlikely that, in vivo, myometrial caveolin expression is under a strong estrogenic control; from the data of Turi et al,²⁵ it also appears unlikely that rat myometrial caveolins during pregnancy are predominantly regulated by estrogen as they report changes in expression at gestations when estrogen levels are unlikely to be dramatically altered. Caveolin-1 is believed to be a protein important for scaffolding signaling complexes at the plasma membrane as well as controlling the activity of these transduction pathways.^{16,17} It is unknown if caveolin-2 has a distinct role(s) from caveolin-1 in smooth muscle but, in other tissues, it has been suggested to play an accessory role to caveolin-1. The role of caveolin-3 in smooth muscle, and whether it differs from that of caveolin-1 or -2, is still unknown. Caveolin-3, however, can induce the formation of caveolae in cell culture systems independently of caveolin-1. A recent suggestion is that caveolin-3, like caveolae, may be inversely proportional to the state of smooth muscle cell differentiation.^{29,30} In addition, caveolin-1 and -2, but not -3, have been co-immunoprecipitated from myometrial tissues with antibodies against maxi-K raising the possibility that caveolin-3 expression and/or subcellular localization may be distinct from caveolin-1 or -2.³¹ The relatively maintained expressions of caveolins in mouse myometrium with pregnancy is consistent with data illustrating no significant change in the caveolin expression, or number of caveolae, in human myometrium with labor onset.^{32,33}

In recent years ROK proteins have been assigned a pivotal role in agonist-dependent Ca^{2+} -sensitization of smooth muscle contraction.^{11–14} Receptor-coupled translocation of the upstream activator of ROK, rhoA, is essential for this process and a direct interaction of rhoA and the scaffolding caveolin domain (at least for caveolin-1) has been suggested in uterine smooth muscle. Here, we report that in mouse myometrium there are gestational-dependent increases in myometrial ROK α and ROK β . Both ROK α : α -actin and ROK β : α -actin ratios were significantly increased on day 19 of pregnancy compared to uteri of NP mice. This is in agreement with a report of gestational-dependent increased ROK

expression, relative to GAPDH, in rat myometrium as is the enhanced actions of the ROK inhibitor Y27632 on myometrial from pregnant, compared to nonpregnant, mice and rats.³⁴ Future analysis of myometrial ROK activity, and efficacy of ROK inhibition, during mid-pregnancy would be informative but the data to date suggest a more prominent role for ROK in mouse uterine contractility as pregnancy progresses.

The thick filament protein MLC₂₀ plays a key role in regulation of myometrial contractility as its phosphorylation by Ca²⁺- and calmodulin-dependent myosin light chain kinase is associated with the onset of uterine contractile effort.^{1–3} In spite of this, however, the expression of MLC₂₀ is significantly depressed in mid- to late pregnancy when compared to myometria from NP mice. Thus, on days 12, 15, 17, or 19 the MLC₂₀: α -actin ratios were almost half those of tissue from NP mice. There may be several possible explanations for this finding. First, a reduced MLC₂₀ expression may guard against premature activation of myometrial contractility. Second, in human myometria at least, contractile activation in samples from pregnant women was associated with a slower rate and extent of MLC₂₀ phosphorylation than tissue from nonpregnant women. This, and the fact that myometrial absolute force production actually increases when MLC₂₀ content has reduced, perhaps reflects less of a reliance on pathways involving MLC₂₀ activation for contractile effort in pregnancy.

The myometrial expression of the thin filament protein *h*-caldesmon has been shown to change with gestation in rat⁶ and humans⁷ and, here, we report a similar increase in *h*-caldesmon, compared to α -actin, in mouse myometria with pregnancy. As *h*-caldesmon is an inhibitor of actomyosin MgATPase activity, its increased expression may reflect a mechanism for limiting uterine contractile activation.³⁵ In this regard it would be expected to play an opposite role to the increased ROK protein expression. As a redistribution of *h*-caldesmon occurs with pregnancy in human myometrium,⁷ an additional possibility is that the enhanced *h*-caldesmon expression may be required of the filamentous rearrangements accompanying myocyte swelling with pregnancy.

In summary, we have found that the expression of myometrial α -actin and caveolins remains relatively constant throughout pregnancy in the mouse. In contrast, other signal transduction (ROK) and contractile filament-associated and (MLC₂₀ *h*-caldesmon) protein expressions were altered with pregnancy. These changes are likely to be related to remodeling of cell architecture and function. Some of these changes—lowered MLC₂₀ and elevated *h*-caldesmon—may be anticipated to limit activation of myometrium. However, the finding that absolute force production of myometrial tissue progressively increased throughout pregnancy, together with elevated ROK expressions and enhanced effectiveness of a ROK inhibitor on myometrial contractions (including rats³⁴), suggests a predominance of contractile activation pathways closer to term. The maintained expression of caveolins may serve the purpose of ensuring a continued presence of a platform for the scaffolding of divergent relaxatory or contractile signaling complexes.

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Figure 1.

Changes in the absolute force of contraction of uterine smooth muscle with pregnancy. The integrated force of contraction per cross-sectional area of myometria of pregnant mice in response to high K^+ stimulation was normalized to that from NP mice. Myometria obtained from mice of gestational days 15, 17, and 19 exhibited significantly enhanced force compared to that of NP mice.

Figure 2. Effect of the estrous cycle on the expressions of α -actin, caveolins, ROKs, MLC₂₀, and *h*-caldesmon in mouse myometria. Representative blots (A and B) showing the expression of proteins of interest in mice from stages of di-estrus, pro-estrus, or estrus.

Figure 3.

Meaned data of the expressions of caveolins, ROKs, MLC₂₀ and *h*-caldesmon relative to α -actin during the estrous cycle of mice. The α -actin content did not vary between the stages of the estrous cycle monitored (A). Subsequently, when compared to the corresponding α -actin contents, there was no significant change in caveolin-1 (B), caveolin-2 (C), caveolin-3 (D), ROK α (E), ROK β (F), MLC₂₀ (G), or *h*-caldesmon (H) in mice from stages of di-estrus, pro-estrus, or estrus.

Figure 4.

Effect of pregnancy on the relative expressions of the caveolins and α -actin in mouse myometria. Caveolin-1 (A), caveolin-2 (D), and caveolin-3 (G) expression, when compared to the corresponding α -actin contents (B, E, H, respectively), showed no significant change in pregnant myometria versus NP mice (C, F, I, respectively).

Figure 5. Relative expressions of ROK α , ROK β , and α -actin in myometria of NP and pregnant mice. Myometrial tissue homogenates from pregnant mice expressed greater amounts of ROK α (A) and ROK β (D; band denoted by the *arrow*) than tissues from NP mice. When compared with α -actin on the same membranes (B and E, respectively) there were significant elevations of the ROK α : α -actin ratio on days 12, 15, 17, and 19 (C), and of the ROK β : α -actin ratio on day 19 (F), relative to NP mice.

Figure 6. MLC₂₀ and α-actin expression in myometria of NP and pregnant mice. Myometrial tissue homogenates from pregnant mice contained significantly less MLC₂₀ (A) than NP mice. Relative to α-actin (B) there was a significant reduction of the MLC₂₀:α-actin ratios on days 12, 15, 17, and 19 compared to NP mice (C).

Figure 7. Relative expressions of *h*-caldesmon and α -actin in myometria of NP and pregnant mice. Myometrial tissue homogenates from day 19 pregnant mice contained significantly more *h*-caldesmon (A) than NP mice. When compared with α -actin (B) there was a significant elevation of the *h*-caldesmon: α -actin ratios on days 17 and 19 relative to NP mice (C).

Figure 8.

Effect of the ROK inhibitor Y-27632 on thromboxane mimetic U46619-induced contractility of NP (A) and late-pregnant (B) mouse myometria. Y-27632 (10 µM) exerted a significantly greater inhibition of U46619-induced (10 µM) contractile amplitude of myometrial strips from pregnant mice compared to NP mice.